EXHIBIT A

THE MIDWINTER CONFERENCE OF IMMUNOLOGISTS
POSTER ABSTRACT - 2005

Name: Deepal Pandya E-mail: dpandya@pdl.com

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Generation of a high affinity humanized anti-IP-10 monoclonal antibody by protein engineering

Deepal Pandya, Alivelu Irrinki, Balaji Balasa, Nicholas F. Landolfi, Shankar Kumar, Paul R. Hinton and Naoya Tsurushita

Protein Design Labs, Inc., 34801 Campus Drive, Fremont, CA 94555 USA

HuAIP12 and HuAIP13 are humanized IgG1/κ monoclonal antibodies derived from independently isolated murine antibodies AIP12 and AIP13, respectively, which bind to and neutralize human IP-10. Although HuAIP12 and HuAIP13 share a high degree of homology in their V region amino acid sequences (there are two amino acid differences in VH and four in Vκ), analyses using competition ELISA and surface plasmon resonance (Biacore) indicated that the binding affinity of HuAIP12 for human IP-10 is higher than that of HuAIP13. Because the humanized antibodies compete with each other for binding to IP-10, it is likely that their parental murine antibodies were derived from common germline VH and VL genes. Mix-and-match analysis of heavy and light chains between HuAIP12 and HuAIP13 indicated that the HuAIP12 VH region is essential for high affinity binding to human IP-10. The HuAIP12 and HuAIP13 VH regions differ only at position 55 (numbered sequentially from the N-terminus of the mature protein) in CDR2 and at position 104 in CDR3. Therefore, each of these positions in the HuAIP12 VH was replaced with the corresponding residue from the HuAIP13 VH (Thr to Ile at position 55, and Gly to Ala at position 104) to identify which of these amino acids is important for the higher affinity of HuAIP12. The substitution in CDR3 reduced the affinity of the variant for IP-10, indicating the importance of Gly at position 104 in the HuAIP12 VH for high affinity binding to IP-10; however, the substitution in CDR2 unexpectedly increased the affinity of this HuAIP12 variant for IP-10 significantly and improved its ability to block IP-10-mediated chemotaxis. This result indicates that Thr at position 55 in the HuAIP12 VH has a negative impact on the binding affinity of HuAIP12 for IP-10. The characteristics of the higher affinity variant make it an excellent candidate for therapeutic testing in autoimmune and inflammatory diseases, such as ulcerative colitis and Crohn's Disease, in which high levels of IP-10 have been associated with disease pathogenesis.

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ABSTRACT

HuAIP12 and HuAIP13 are humanized IgG1/κ monoclonal antibodies derived from independently isolated murine antibodies AIP12 and AIP13, respectively, which bind to and neutralize human IP-10. Although HuAIP12 and HuAIP13 share a high degree of sequence homology (four amino acid differences in VL and two in VH) and recognize the same epitope on human IP-10, HuAIP12 binds better than HuAIP13 to IP-10. Mix-and-match analysis of the heavy and light chains between HuAIP12 and HuAIP13 indicated that the HuAIP12 VH region is essential for high affinity binding to human IP-10. Since the HuAIP12 and HuAIP13 VH regions differ only at two positions, each of these positions in the HuAIP12 VH was replaced with the corresponding residue from the HuAIP13 VH to study the contributions of these amino acids to the higher affinity of HuAIP12. The substitution of Gly to Ala at position 104 in CDR3 reduced the affinity of the variant for IP-10; however, the substitution of Thr to Ile at position 55 in CDR2 unexpectedly increased the affinity of this HuAIP12 variant for IP-10 significantly and improved its ability to block IP-10-mediated chemotaxis. The characteristics of the higher affinity variant make it an excellent candidate for therapeutic testing in diseases where IP-10 plays a role in pathogenesis.

RESULTS

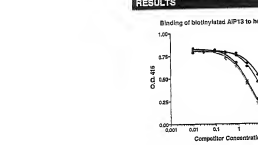

Figure 1. Competitive binding of humanized anti-IP-10 antibodies and hybrid antibodies to human IP-10. The binding of biotinylated murine AIP13 antibody (0.5 pg/ml) to human IP-10 in the presence of increasing concentrations of purified HuAIP12, HuAIP13, HuAIP13 heavy chain + HuAIP12 light chain (13H + 12L) or HuAIP12 heavy chain + HuAIP13 light chain (12H + 13L) competitor antibodies was detected with streptavidin conjugated HRP and analyzed with a spectrophotometer.

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                                                    30
HuAIP12          EVQLVQSGAEVKKPGATVKISCKVSGYTFT
HuAIP13          EVQLVQSGAEVEKPGATVKISCKVSGYPFT

                                                    60
HuAIP12          DYFHWVRQAPGKGLKWMGWINTETGEPTY
HuAIP13          DYFHWVRQAPGKGLEWMGWINTEIGEPTY

                                                    90
HuAIP12          ADDFKGRFVFTLDTSTSTAYMELSSLRSED
HuAIP13          ADDFKGSFVFTLDTSTSTAYMELSSLRSED

                                                   119
HuAIP12          TAVYYCARNYDYDGYFDYWGQGTTVTVSS
HuAIP13          TAVYYCARNYDYDGYFDYWGQGTTVTVSS
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Figure 2. Alignment of the VH amino acid sequences of HuAIP12 and HuAIP13. The amino acid sequences of the mature VH region are shown in single letter code. The CDRs based on the definition of Kabat are in bold green letters. The amino acids that are different between HuAIP12 and HuAIP13 (positions 55 and 104) are shown in bold red letters. The sequences are numbered sequentially from the N-terminus.

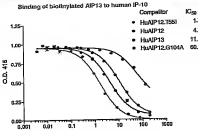

Figure 3. Competitive binding of HuAIP12, HuAIP13 and mutant HuAIP12 antibodies to human IP-10. The binding of biotinylated murine AIP13 antibody (0.5 μg/ml) to human IP-10 in the presence of increasing concentrations of purified HuAIP12, HuAIP13, HuAIP12.T55I or HuAIP12.G104A competitor antibodies was detected with streptavidin conjugated HRP and analyzed with a spectrophotometer.

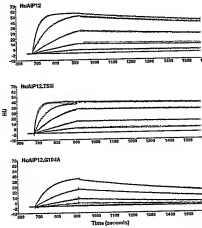

Figure 4. Biacore analysis of HuAIP12 wild-type and variant antibodies. The affinities of the HuAIP12 wild type, T55I and G104A antibodies to human IP-10 were characterized by the surface plasmon resonance method using a Biacore 2000 apparatus. Goat anti-human IgG, γ chain specific antibody (GAHFc) was immobilized on a CM5 sensor chip by amine coupling. HuAIP12 antibodies were captured with GAHFc on the surface of a CM5 sensor chip. Human IP-10 at concentrations ranging from 0.34 nM to 83.3 nM was then injected. Data analysis was carried out using BIAevaluation software.

Table 1. Affinity of HuAIP12 and variants to human IP-10 measured using the surface plasmon resonance method.

HuAIP12	ka (1/Ms)	kd (1/s)	KD (M)
Wild type	1.55×10^{6}	3.81×10^{-4}	2.51×10^{-10}
T55I	1.66×10^{6}	4.67×10^{-5}	2.69×10^{-11}
G104A	1.31×10^{6}	7.84×10^{-4}	5.98×10^{-9}

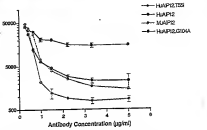

Figure 5. Analysis of IP-10-mediated chemotaxis. The ability of anti-IP10 antibodies to block IP-10-mediated chemotaxis of CXCR3 expressing Ba/F3 cells was tested. Human IP-10 (125 ng/ml) was mixed with increasing concentrations of anti-IP-10 antibodies. Migration of CXCR3-Ba/F3 cells through a membrane towards a chamber containing IP-10 was carried out for 1.5 hours. Migrated cells were labeled with CellTiter-Glo and luminescence was measured with a Lumicount plate reader.

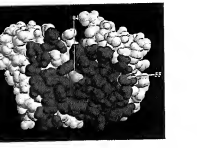

Figure 6. Three-dimensional structure model of the murine AIP12 variable region. Framework residues are white and CDR residues are red. Amino acids at positions 55 and 104 in the VH are cyan and yellow, respectively.

CONCLUSIONS

- The VH region of HuAIP12 is essential for maintaining its high affinity binding to human IP-10 (Fig. 1).
- Gly at position 104 in the HuAIP12 VH is important for retaining high affinity binding to human IP-10 (Fig. 3).
- Thr at position 55 in the HuAIP12 VH has a negative impact on the binding affinity to human IP-10 (Fig. 3).
- The affinity of the HuAIP12.T55I variant for human IP-10 (0.0269 nM) is ~10-fold higher than that of wild type HuAIP12 (Table 1).
- The higher affinity of the HuAIP12.T55I variant is attributed to its slower off-rate compared with wild type HuAIP12 (Table 1).
- HuAIP12.T55I is more effective in blocking IP-10-mediated chemotaxis compared with wild type HuAIP12 (Fig. 5).
- HuAIP12.T55I is an excellent candidate for therapeutic testing in autoimmune and inflammatory diseases, such as ulcerative colitis and Crohn's disease, in which high levels of IP-10 have been associated with disease pathogenesis.

EXHIBIT B